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Single particle analysis of thylakoid proteins from *Thermosynechococcus elongatus* and *Synechocystis* 6803: Localization of the CupA subunit of NDH-1

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Abstract **The larger protein complexes of the cyanobacterial photosynthetic membrane of *Thermosynechoccus elongatus* and *Synechocystis* 6803 were studied by single particle electron microscopy after detergent solubilization, without any purification steps. Besides the "standard" L-shaped NDH-1L complex, related to complex I, large numbers of a U-shaped NDH-1MS complex were found in both cyanobacteria. In membranes from *Synechocystis* Δ*cupA* and Δ*cupA*/*cupB* mutants the U-shaped complexes were absent, indicating that CupA is responsible for the U-shape by binding at the tip of the membrane-bound arm of NDH-1MS. Comparison of membranes grown under air levels of CO_2 or 3% CO_2 indicates that the number of NDH-1MS particles is 30-fold higher under low-CO_2.**

Keywords: Electron microscopy; Single particle analysis; Membrane protein; NDH-1; CupA; *Thermosynechococcus elongatus*; *Synechocystis* 6803

1. Introduction

Over the last 20 years, single particle analysis of electron microscopy (EM) projections has become a well-established technique to obtain structural information about large biomacromolecules at a resolution of 10–20 Å [1]. In some favourable cases a much higher resolution has been achieved on ice-embedded virus particles or other complexes with a high symmetry [2]. Membrane proteins are more difficult to process by cryo-EM at high resolution, because single complexes are surrounded by a detergent layer to keep them in a monodisperse state. Single particle averaging on negatively stained specimens, however, offers the possibility to process thousands of projections within a day, yielding 2D projection maps of at least 20 Å resolution. The method includes statistical analysis and classification, and is able to sort and average 2D projection maps of membrane proteins from heterogeneous samples, which can be semi-purified samples. In this way, a set of projection maps is achieved, which may contain the object of interest, but also maps specific contaminants. In a recent study of photosystem II (PSII) complexes from cyanobacteria two different particles, obtained by different purification schemes, were compared by single particle analysis, SDS gel analysis and mass spectrometry [3]. Mass spectrometry indicated that the two particles differed by the absence or presence of PsbZ, a small peripheral subunit of 6.8 kDa. By EM analysis the location within the structure could be determined [3]. In addition, a contaminating L-shaped protein was found, which was suggested to be NDH-1, the cyanobacterial counterpart of Complex I. This tentative assignment of NDH-1 could be confirmed after comparison with purified NDH-1 [4].

Arteni and coworkers suggested that screening for membrane protein structures by single particle EM, in combination with biochemical analysis, might be an interesting approach to find novel protein structures [3]. To further test the idea of finding novel transient membrane protein complexes by single particle EM we applied the method to a complete set of non-purified complexes from solubilized photosynthetic membranes of two different cyanobacteria: *Thermocynechococcus elongatus* and *Synechocystis* 6803 (see below). One of the aims was to find novel structures, for instance of NDH-1, because this is a very fragile, heterogeneous complex, see [5] for a review.

Proteomic studies revealed that the cyanobacterium *Synechocystis* 6803 has several functionally distinct NDH-1 complexes [5,6]. The two largest complexes, NDH-1L and NDH-1MS, have a mass of about 500 kDa. The NDH-1L complex participates in respiration and cyclic electron flow around PSI. It is composed of 15 subunits, of which eight belong to the membrane domain (NdhA, NdhB, NdhC, NdhD1, NdhE, NdhF1, NdhG and NdhL) and seven to the hydrophilic domain (NdhH-J, NdhM–NdhO) [7]. NDH-1L appeared relatively unaffected by different growth conditions such as high- and low-CO_2, iron deficiency, salt stress. The NDH-1MS complex functions as a high-affinity CO_2 uptake system and converts CO_2 to HCO_3^- within the cell. It is strongly expressed in cells cultured under air levels of CO_2, but the expression is drastically reduced under increased CO_2 levels [8]. The two NDH-1 complexes differ in subunit composition. In the

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Abbreviations: HC, high-CO_2 (3% or 5%); LC, low-CO_2 (air level)

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NDH-1MS complex the D1 subunit of 56 kDa and the F1 subunits of 72 kDa have been replaced by homologous but slightly smaller D3 (54 kDa) and F3 (66 kDa) subunits, which are essential for active CO_2 uptake [9]. In addition, NDH-1MS has two water-soluble proteins attached. One is CupA, a 51 kDa protein, which was found to be a component of NDH-1 involved in CO_2 uptake by reverse genetics studies [10,11] and the other is a small subunit recently named CupS [12] and encoded by *sll1735* in *Synechocystis* 6803 and *tll0221* in *T. elongatus*. There is in addition a second NDH-1 particle functionally in CO_2 uptake; it has been designated NDH-1MS′ [5]. This particle of 440 kDa has subunits D1 and F1 replaced by D4 and F4 and has CupB attached, which is a homologue of CupA, and CupS [10,11]. It appears that the NDH-1MS′ particle shows low affinity to CO_2 and is constitutively expressed, in contrast to the high-CO_2 affinity NDH-1MS complex, which is induced under low-CO_2 [10].

In this paper, we analyzed the larger membrane- and membrane associated complexes from *T. elongatus* and *Synechocystis* 6803 by single particle EM analysis directly after solubilization, without a purification step, to omit potential loss of transiently bound subunits. We focussed on NDH-1 complexes, which can be easily detected in EM images, because of the unique L-shape. In addition to L-shaped NDH-1, we found U-shaped particles, which had a similar hydrophilic domain as the L-shaped NDH-1 complex named NDH-1L [4]. To establish the presence of CupA in the extra domain of the U-shaped particles, we made a comparison to particles solubilized from the membranes of CupA and CupA/B mutants and here we show that the loosely attached density at the tip of the membrane arm of NDH-1 particles is identical to the CupA protein.

2. Materials and methods

2.1. Cultivation of cyanobacteria and preparation of thylakoid membranes

Synechocystis sp. PCC 6803 WT, Δ*cupA* and Δ*cupA*/*cupB* strains were grown in BG-11 medium under continuous light of 50 μmol photons m^{-2} s^{-1} at 3% CO_2 (HC), and then transferred to air level of CO_2 (LC). The thylakoid membranes of *Synechocystis* WT and mutant strains were isolated from 100 ml cell culture according to Ref. [13].

Thermosynechococcus elongatus WT was cultured at 45 °C in BG-11 media at 5% CO_2 (HC) or air level of CO_2 (LC) and illuminated with white light of increasing intensity (50–250 μmol photons m^{-2} s^{-1}). Thylakoid membranes were prepared according to Ref. [14].

2.2. Solubilization of membranes from T. elongatus and Synechocystis 6803

For preparing EM specimens, membranes from *T. elongatus* grown at high (5%)-CO_2 and low-CO_2 were solubilized with 1% (or 2%) digitonin at a 0.5 mg/ml final chlorophyll concentration. The suspension was stirred for 30 min using a small magnet stirrer, at 4 °C and followed by 15 min centrifugation at 15000 g. The unsolubilized material was discarded and the supernatant used for electron microscopy analysis. Thylakoid membranes from *Synechocystis* WT and mutants were solubilized using digitonin at 3% final concentration and 0.3 μg/μl chlorophyll final concentration for all samples (WT, ΔcupA, ΔcupA/B grown in low-CO_2 and high-CO_2).

For blue-native (BN)/SDS–PAGE, the thylakoid membranes of *Synechocystis* WT, Δ*cupA* and Δ*cupA*/*cupB* (7.5 μg chlorophyll) were pelleted down and resuspended at a chlorophyll concentration of 0.6 μg/μl. Then an equal volume of detergent solution was added to the thylakoid suspensions to final detergent concentration of 1.5% β-DM or 3% digitonin (the final chlorophyll concentration 0.3 μg/μl), and the gels were run for 5 h and stained with silver as described earlier [13]. For immunoblotting, thylakoids equivalent to 3 μg chlorophyll were solubilized and separated in SDS–PAGE containing 6 M urea [15]. The proteins were electrotransferred to a PVDF membrane and immunodetected by specific antibodies against CupA, NdhD3 and AtpA/B proteins [13]. The AtpA/B antibody was kindly provided by Dr. Hundal.

2.3. Electron microscopy and single particle analysis

Samples were made from solubilized membranes by dilution in buffer with detergent and subsequent negative staining using 2% uranyl acetate on glow-discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM120 equipped with a LaB_6 tip operating at 120 kV. The "GRACE" system for semi-automated specimen selection and data acquisition [16] was used to record 2048 × 2048 pixel images at 66850× calibrated magnification (3.75 Å) with a Gatan 4000 SP 4K slow-scan CCD camera. A total of 15000 particle projections were collected. Single particle analysis was performed using Groningen Image Processing ("GRIP") software packages on a PC cluster. The best 70–80% of the class members was taken for the final class-sums.

3. Results

3.1. Structural analysis of solubilized membranes

Thylakoid membranes of wild-type *T. elongatus* and *Synechocystis* 6803, grown at low-CO_2 and high-CO_2 conditions were solubilized with 1–3% digitonin and directly applied to electron microscopy grids without further purification steps. A typical EM image of negatively stained particles is presented in Fig. 2. From such images we selected all large recognizable single particle projections. They were analyzed by single particle averaging, which includes classification of projections and the averaging of class-members of homogenous subsets of particles into 2D maps. A gallery of selected 2D maps is presented in Fig. 2. First, we found a novel U-shaped complex in top- and side-view position (Figs. 2A and B). The upper left part strongly resembles the hydrophilic domain of purified NDH-1 from *T. elongatus* in side-view position (Fig. 2C) [4], which indicates that this U-shaped particle is a NDH-1 complex.

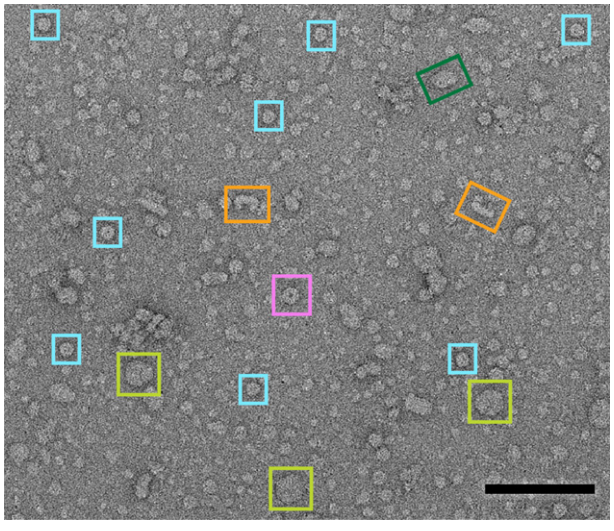

Fig. 1. EM frame showing an overview of solubilized membranes from *Thermocynechococcus elongatus*, grown on high-CO_2. Boxes mark projections of trimeric Photosystem I (bright green); dimeric photosystem II (dark green), phycobilisome fragments (blue), NDH-1 (orange), and an unknown hexagonal particle (purple). The space bar equals 100 nm.

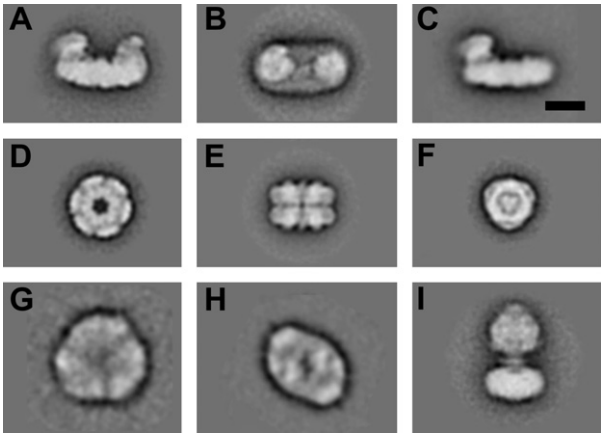

Fig. 2. A gallery of 2D projection maps from single particle EM of solubilized membranes from *T. elongatus* and *Synechocystis* PCC 6803. (A) NDH-1 side view from *T. elongatus*, (B) NDH-1 top view from *T. elongatus*, (C) purified NDH-1 from *Synechocystis* (reproduced from [4]), (D and E) a water-soluble hexagonal particle, tentatively assigned to glutamine synthetase in top- and side-view position, (F) cyanobacterial fragment with trimeric symmetry assigned to allophycocyanin, (G) trimeric photosystem I complex, (H) Photosystem II dimeric complex from *Synechocystis*, (I) proton ATP synthase complex. Space bar for all frames equals 10 nm.

Identical complexes were found in solubilized membranes from *Synechocystis* 6803. Second, we found a hexagonal particle, in top- and side-view position was very common after solubilization, even if the membranes were washed several times to remove water soluble components (Fig. 2D and E). The staining profile suggests that it probably is a membrane-attached protein rather than a membrane-integral complex, because the latter always have some low-contrast areas due to the fact that the negative stain is not penetrating hydrophobic domains of proteins. It is tentatively assigned to glutamine synthetase. A cyanobacterial fragment with trimeric symmetry is assigned to allophycocyanin (Fig. 2F), because very similar particles were observed from fragmented phycobilisomes (A.A. Arteni, E.J. Boekema, unpublished observations). Next, the "standard" trimeric Photosystem I (PSI) complex (Fig. 2G) and the dimeric PSII complex (Fig. 2H) were common in both cyanobacteria and finally, the proton ATP synthase complex (Fig. 2I) was found. In addition, small numbers of double dimers of PSII were found (not shown), which were described before in Ref. [17].

3.2. Comparison of WT and mutant Synechocystis membranes

The U-shaped particle found by analyzing the major complexes from solubilized, non-purified membranes of *T. elongatus* is a NDH-1 complex, because of similarities to purified L-shaped NDH-1 from *T. elongatus* [4], see Fig. 1. The question then remains is how to assign the extra mass on the tip of the hydrophobic arm. For this purpose, we analyzed in addition the membranes from *Synechocystis* CupA and CupA/B mutants and compared particles with those of wild-type *Synechocystis* 6803 membranes. They were grown at low- and high-CO_2 levels, because of possible differences of expression of NDH-1 particles. From each solubilized membrane probe around 500 images were recorded and on average, about 1000 single particles were selected for single particle analysis. Statistical analysis and classification showed that in all preparations U-shaped and/or L-shaped particles were present but

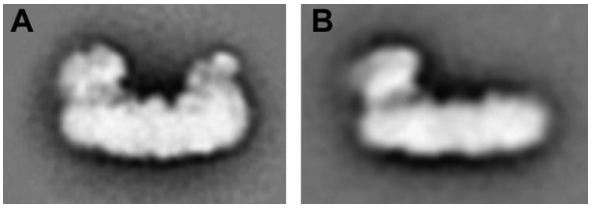

Fig. 3. Comparison of NDH-1 complexes. (A) The U-shaped NDH-1 complex from *T. elongatus* with the standard NDH-1L complex purified with β-DM. (B) The extra density on the tip of the hydrophobic arm is assigned to CupA (see text).

within the 2D maps of these particles no variation was found (Fig. 3). There were however, significant differences in the numbers of particles. The electron microscopy specimens were made by diluting the solubilized material a hundred times. In this way the total amount of solubilized membrane complexes was comparable on all specimens. Hence, we could estimate the relative amount of NDH-1 by counting numbers of particles per image. Table 1 shows that in the case of WT the total amount (U-shaped and L-shaped) NDH-1 is three times higher in low-CO_2 compared to high CO_2. If we take into account that the ratio of L-shaped to U-shaped particles was 3.5:6.5 in low-CO_2 (see Table 1) this means under low- and high CO_2 conditions the amount of L-shaped particles is the same (within about 10%), but that the amount of U-shaped particles is about 20 times higher under low-CO_2 conditions.

In the case of mutants, the amount of NDH-I is low compared to the wild-type. In all these cases only L-shaped Complex I could be observed. In conclusion, according to electron microscopy data the total absence of U-shaped particles in the Cup A mutant and the CupA/CupB double mutant indicates that the extra mass at the tip of the hydrophobic arm is related to CupA.

In wild-type *T. elongatus* there was also a difference in the ratio of U-shaped and L-shaped particles; there were significantly more U-shaped particles under low-CO_2 conditions, confirming the semi-quantitative results in *Synechocystis* 6803.

3.3. Biochemical characterization of Synechocystis WT, ΔcupA and ΔcupA/cupB membranes

The composition of *Synechocystis WT*, ΔcupA and ΔcupA/cupB membranes was compared by performing BN–PAGE and SDS–PAGE (Fig. 4). The membrane of ΔcupA/cupB

Table 1
Numbers of L-shaped and U-shaped NDH-1 complexes found after detergent solubilization in *Synechocystis* WT and mutants grown at high and low CO_2 conditions

High CO_2		Low CO_2	
Number of particles		Number of particles	
771 (590 images)		2500 (834 images)	
WT Synechocystis			
35 U-shaped	736 L-shaped	1624 U-shaped	876 L-shaped
119 (280 images)		360 (439 images)	
Synechocystis CupA mutant			
0 U-shaped	119 L-shaped	0 U-shaped	360 L-shaped
350 (500 images)		800 (921 images)	
Synechocystis CupA/B mutant			
0 U-shaped	350 L-shaped	0 U-shaped	800 L-shaped

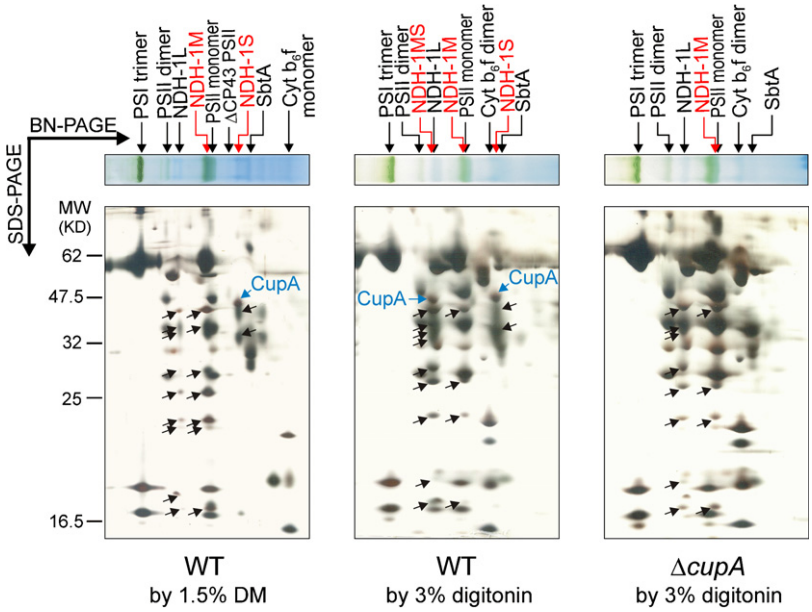

Fig. 4. BN–PAGE gels (on the top) and silver stained 2D-BN/SDS–PAGE gels of *Synechocystis* WT and Δ*cupA* membranes. The cells were grown at low CO_2 (LC) conditions, and the membranes were solubilized with 1.5% DM or 3% digitonin. The major protein complexes on BN gels are marked. The NDH-1M, -S and -MS complexes are emphasized in red. In silver stained 2D gels the black arrows indicate the Ndh subunits in various NDH-1 complexes (NDH-1L, NDH-1M, NDH-1MS and NDH-1S). Note that the NDH-1MS complex is present only in WT thylakoids solubilized with digitonin, and harbors about half of the CupA proteins, the other half residing in the NDH-1S complex. The CupA protein is indicated by blue arrows. *Notes*: SbtA: the Na^+-dependent primary bicarbonate (HCO_3^-) transporter; NDH-1S, a fragment of NDH-1MS, containing NdhD3, NdhF3, CupA and CupS proteins.

was very similar to that of ΔcupA (data not shown). The 2D gels clearly demonstrated that NDH-1MS (containing CupA protein) was totally dissociated to NDH-1M and NDH-1S in DM solubilized WT low-CO_2 sample, whereas about half of NDH-1MS remained intact in digitonin solubilized WT (low-CO_2) sample. Due to similar molecular masses of NDH-1L and NDH-1MS, these two complexes migrated at almost the same position in BN-PAGE (Fig. 4). However, the NDH-1MS complex can be distinguished by the presence of the CupA protein (48 kDa), and by a slightly bigger mass than NDH-1L. The mutants ΔcupA (Fig. 4) and ΔcupA/cupB (data not shown) lack completely CupA containing NDH-1S and NDH-1MS complexes. Comparing the samples solubilized by these two detergents at conditions used here, it is also clearly shown that cytochrome b6f complex was in its dimer form when digitonin was used to solubilize the membranes, while mainly as monomer when using DM. This indicated that 3% digitonin solubilized membranes are more intact compared to those using 1.5% DM.

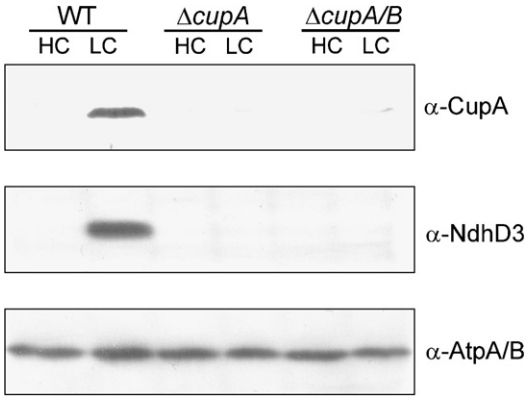

Fig. 5. Immunoblots of *Synechocystis* WT, Δ*cupA* and Δ*cupA*/*cupB* membranes demonstrating that the CupA and NdhD3 proteins are present only in the WT low CO_2 sample and missing from all other samples. Numbers of L-shaped and U-shaped NDH-1 complexes found after detergent solubilization in *Synechocystis* WT and mutants grown at high- and low-CO_2 conditions.

It seems that about half of the NDH-1MS complex remains intact in 3% digitonin solubilized membranes, which corresponds to U-shaped particles in EM. In DM solubilized samples, the NDH-1MS complex was totally dissociated to subcomplexes. The CupA containing NDH-1 complexes were completely missing in the CupA and CupA/B mutants.

The immunoblots (Fig. 5) clearly demonstrate that the CupA and NdhD3 proteins, which belong to the NDH-1MS complex were present only in WT low-CO_2 sample, and were missing from high-CO_2 samples as well as from the Δ*cupA* and Δ*cupA*/*cupB* mutants at low-CO_2.

4. Discussion

The aim of this work was to show that single particle analysis is able to find novel structures of membrane proteins or protein complexes. In the case of the photosynthetic membranes of *T. elongatus* several novel structures were found, including a U-shaped NDH-1 particle. The omission of a purification step is crucial, because only very low numbers of U-shaped NDH-1 complexes were found in an EM analysis of purified NDH-1 from *T. elongatus* [4]. The assignment of the composition of the extra domain, however, can only be solved in combination with other methods. There are at least three strategies to solve this problem. The first is to purify the NDH-1 complex by chromatography and to perform mass

spectrometry. Because it is known that NDH-1 from some cyanobacteria such as *T. elongatus* has a His-rich region in the membrane-integral F1 subunit [18], this could be used as a natural His-tag for affinity purification. However, NDH-1 complexes are very labile, as mentioned before. The second strategy is to compare with one of the several cyanobacteria that lack genes that may be responsible for the extra domain. There are several cyanobacteria without the CupA and/or CupB gene in the genome [12].

The third strategy, applied in this study, is to work with mutants that lack CupA. In the membranes of the ΔCupA and ΔCupA/B mutants no U-shape particles were found (Table 1), which indicates that CupA is located at the tip of the membrane arm of NDH-1.

The general conclusion of our EM investigation, in combination with existing data on the heterogeneity of NDH-1, is that there are two main NDH-1 complexes in *T. elongatus* and *Synechocystis* 6803. First, there is a L-shaped NDH-1L complex, which is expressed in equal numbers under low- and high-CO_2 levels and second, there is a U-shaped NDH-1MS complex, which is expressed in high numbers under low-CO_2levels. In this complex, CupA is attached to the tip of the membrane arm. This conclusion is confirmed by BN–PAGE/SDS–PAGE experiments on the same mutants (Fig. 4). In EM side view projection, the area of the extra domain at the tip of the membrane arm is 59% of the 49 nm^2 area of the hydrophilic arm, which is composed of the subunits NdhH, I, J, K, M, N and O with a total mass of 170 kDa [7]. From 2D surfaces the 3D volume can only be roughly estimated because the third dimension is not known. But if the CupA protrusion would have the same proportions in 3D as the hydrophilic arm, the unknown third dimension could be about 5.5 nm and the extra domain would have a mass of up to 80 kDa. This is compatible to 61 kDa, the sum of single copies of CupA (51 kDa) and CupS, a 10 kDa protein proposed to associate to CupA [5].

The role of CupB is not yet established. CupB might be associated to NDH-1MS′ in a similar way as CupA to NDH-1MS [5]. It was proposed that the NDH-1MS′ particle shows low affinity to CO_2 and is constitutively expressed, in contrast to the high-CO_2 affinity NDH-1MS complex, which is induced under low-CO_2 [10]. However, in the CupA mutant there were no remaining U-shaped particles present. Moreover, if there would have been two types of U-shaped particles in the WT membranes of *Synechocystis*, it is very likely that the EM projection maps would differ slightly because the Cup B domain at the tip of the membrane arm is about 20 kDa smaller than the CupA plus CupS domain. However, such a heterogeneity was not found. Another particle that might have been expected is the NDH-1 particle with the extended hydrophilic arm. The standard cyanobacterial and chloroplast NDH-1 complexes are very similar to the *E. coli* Complex I, but lack counterparts of the NuoE, -F and -G subunits, which results in a substantial shorter arm than the complex I from *E. coli* (see Ref. [19] for a review). However, low numbers of NDH-1 complexes with an extended arm were found in an analysis of a large dataset of purified NDH-1 complexes from *T. elongatus* [4], indicating NDH-1 particles including proteins with a structural homology to the *E. coli* Complex I NuoE, -F and -G subunits. In the current study such particles were almost not observed, which indicates that this catalytic domain is not present in significant numbers. It might be more abundant under other growth conditions, such as heterotrophic growth.

In summary, our combined approach of electron microscopy and biochemical analysis shows that it is possible to assign a transient component of a multi-subunit protein complex without a single purification step, if averaged 2D projection maps can be obtained with enough structural resolution to directly assign them to a specific protein complex.

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